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Prolonged ethanol administration prevents the development of tolerance to morphine-induced respiratory depression

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ABSTRACT

Background

Opioid users regularly consume other drugs such as alcohol (ethanol). Acute administration of ethanol can rapidly reverse tolerance to morphine-induced respiratory depression. However, alcohol consumption by opioid users is likely to occur over prolonged time periods. We have therefore sought to determine the effect of prolonged alcohol consumption on the development of tolerance to opioid respiratory depression.

Methods

Mice were fed control or ethanol (5%) liquid diet for 16 days. On days 9-16 morphine tolerance was induced by administration of 3 priming injections of morphine followed by subcutaneous implantation of a morphine-filled osmotic mini-pump. Control mice received saline. Respiration was measured by plethysmography and the effect of an acute morphine challenge dose was measured on day 16 to assess the development of morphine tolerance.

Results

Prolonged ethanol consumption for 14 days did not alter the respiratory depressant effect of an acute dose of morphine. Control mice treated with prolonged morphine developed tolerance to acute morphine respiratory depression whereas ethanol diet fed mice treated with prolonged morphine showed significant respiratory depression during morphine-pump treatment and remained sensitive to the respiratory depressant effect of the acute challenge dose of morphine. The ethanol consumption did not alter blood or brain levels of morphine, whilst conversely prolonged morphine treatment did not alter blood levels of ethanol.

Conclusions

Prolonged ethanol consumption prevents the development and maintenance of tolerance to the respiratory depressant effect of morphine. These data suggest that ethanol inhibition of tolerance will greatly increase the risk of fatal heroin overdose in humans.

1. INTRODUCTION

Heroin users are notorious poly drug users and often take other drugs such as cocaine, alcohol (ethanol), benzodiazepines, amphetamine and gabapentinoids in addition to heroin or other opioids (Darke and Hall, 2003; Borriello et al., 2014; Preston et al., 2016; Lyndon et al., 2017; Wang et al., 2017; Moses et al., 2018; Palamar et al., 2018). An important question is how use of these other drugs might alter the risk of opioid overdose. Death in acute opioid overdose results primarily from respiratory depression (Pattinson, 2008; Montandon and Slutsky, 2019), although in some instances inhalation of vomit may also be a factor. With a depressant such as ethanol it is generally assumed that its effect will simply be additive with that of an opioid, increasing the level of respiratory depression thus making overdose more likely. However, the levels of ethanol found in heroin overdose deaths are rarely high (Darke and Hall, 2003; Hickman et al., 2007; Shah et al., 2008; Green et al., 2011; Fugelstad et al., 2014) which may indicate some other form of interaction other than simple additivity (Hickman et al., 2008).

We have previously reported that in mice acute, low doses of ethanol rapidly reverse tolerance to the respiratory depressant and analgesic effects of morphine and oxycodone (Hull et al., 2013; Hill et al., 2016; Jacob et al., 2017; Hill et al., 2018) but do not reverse tolerance induced by methadone (Hill et al., 2016). Reversal of morphine tolerance by a low concentration of ethanol (20 mM) can also be observed at the single neuron level *in vitro* (Llorente et al., 2013; Jacob et al., 2018) indicating that the effect is at the level of cells expressing μ-opioid receptors to reverse receptor desensitization, rather than an action to alter drug disposition in the brain.

Although acute ethanol administration reversed morphine tolerance in animal studies, heroin users are likely to drink alcohol over prolonged periods of time and this may produce a different interaction given that prolonged exposure to ethanol is known to exert a plethora of effects on enzyme and regulatory protein activity or expression (Elves et al., 1984; You et al., 2002; Sivapiriya et al., 2006) as well as receptor regulation (Fischer et al., 2003; Gustot et al., 2006; Nagy, 2008). Therefore, in the present study we have examined the effect of prolonged ethanol consumption on the acute respiratory depressant response to morphine and on the subsequent induction and maintenance of tolerance to morphine. To do this we fed mice on a diet containing ethanol (Bertola et al., 2013) prior to exposing them to morphine and observed how the exposure to ethanol affected the acute response morphine and the level of tolerance that developed to morphine-induced respiratory depression. Prolonged exposure to ethanol did not alter the respiratory depressant effect of an acute dose of morphine but was found to prevent the development of tolerance to morphine respiratory depression.

2 METHODS AND MATERIALS

2.1 Animals

Male CD-1 mice (Charles River, UK) weighing approximately 30 g (at the beginning of the experiment) were maintained at 22 °C on a reversed 12 h dark-light cycle with food and water available *ad libitum* prior to experimental diet administration. All experiments were performed in the dark (active) phase. Mice were randomly ascribed to treatment groups with the experimenter blinded to the drug treatment. All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986, the European Communities Council Directive (2010/63/EU) and all protocols were approved by the University of Bristol's Animal Welfare and Ethics Review Board.

2.2 Administration of ethanol diet

Mice were housed in groups of 4. Each cage had a single ball bearing feeder filled with ethanol (5%) or control liquid diet. Diets were made up fresh each morning and swapped with the previous day's feed before the dark (active) cycle began. Diet consumption was measured every day and animal weight was measured every third day. Lieber Decarli '82 ethanol diet was procured from (Bio-Serv, USA) and made up according to previously published guidelines (Bertola et al., 2013).

Each cage was provided with 150 ml of control or ethanol liquid diet each day which was approximately double the volume expected to be consumed (Bertola et al., 2013), thus ensuring that mice always had access to the liquid diet. Control diet was made by combining 114 g of control diet mix with water up to a total volume of 500 ml. Ethanol diet was made by combining 66.5 g of ethanol diet mix, 10.2 g of maltose dextrin, and 26.3 ml of ethanol. This was made up to a total volume of 500ml with water. Maltose dextrin was used to calorie balance the ethanol diet with the control diet. Diets were thoroughly mixed to ensure all ingredients were fully dissolved and to prevent blockages of the feeding bottles. Additional access to water is not required when mice are fed on liquid diets and thus was not provided (Bertola et al., 2013). Diet consumption was measured as weight consumed each day per cage and divided by the number of mice per cage to calculate average diet consumption per mouse per day. All cages were observed to have excess liquid diet remaining in unclogged feeders, indicating that the amount of diet provided was in excess of that consumed and that access had not been curtailed by any blockage in the feeder.

2.3 Measurement of respiration

Respiration was measured in freely moving mice using plethysmography chambers (EMKA Technologies, France) supplied with a 5% CO_2 in air mixture (BOC Gas Supplies, UK) as described previously (Hill et al., 2016). Rate and depth of respiration were recorded and converted to minute volume. In some experiments the change in minute volume following acute drug administration for each mouse was calculated as the percentage of the pre-drug baseline. Presenting data as percentage change from the pre-drug levels has been performed to control for variation between treatment groups that may have different baseline levels of respiration.

2.4 Prolonged morphine treatment

After 8 days of exposure to control or ethanol liquid diet prolonged morphine treatment was initiated by first administering three priming injections of morphine (100 mg/kg i.p.) at 12 h intervals followed by subcutaneous implantation on the dorsal flank of an osmotic mini-pump (ALZET®) containing 56.25 mg/ml morphine (to deliver 45 mg/kg/day) for a further 6 days. The dose of morphine that can be delivered from an osmotic minipump is limited by its solubility. For this reason, priming injections of morphine were used to initiate the development of tolerance and tolerance was them maintained by a lower, but continuous release of morphine from the minipump. This method of inducing and maintaining tolerance to various opioid agonists has previously been studied by ourselves and others (Quillinan et al., 2011; Hill et al., 2018; Hill et al., 2016). Control mice received saline injections and implantation of an osmotic mini-pump containing saline. Implantation of osmotic mini-pumps was done under brief (<5 min) isoflurane general anesthesia.

2.5. Assessment of opioid tolerance

To assess the level of tolerance induced by prolonged morphine treatment, mice were injected with an acute challenge dose of morphine (10 mg/kg i.p.) on the final day of the experiment and respiration monitored for 30 min. The degree of respiratory depression observed in prolonged morphine-treated mice was compared to that observed in saline-treated mice which had also been administered an acute challenge dose of morphine (10 mg/kg i.p.).

2.6. Measurement of ethanol and morphine levels

At the end of the experiment (day 16), mice were exsanguinated following death by escalating CO_2 administration. Blood was then collected from the descending abdominal aorta. Syringes used for the collection of blood samples were pre-filled with 0.1ml of heparin (200

units/ml). 100 µl of each plasma supernatant was mixed thoroughly with 500 µl acetonitrile containing 200 ng/ml of deuterated morphine as internal standard and centrifuged at 13 000 r.p.m. for 10 min at room temperature. Additional 100 µl volumes of each plasma supernatant was mixed thoroughly with 500 µl of pure acetonitrile for analysis of ethanol levels. 300 µl samples of the supernatant were evaporated to dryness using a speed vac.

Immediately after collection of blood samples, the brains of the mice were removed and flash frozen in liquid nitrogen. Brains were homogenized in phosphate buffer solution added at a ratio of 2 ml per gram of brain matter. 100 µl of aliquots of brain homogenate samples were mixed thoroughly with 500 µl acetonitrile containing 200 ng/ml of deuterated morphine as internal standard and extracted as described for plasma samples.

For morphine determination plasma and brain samples were analyzed as follows. Samples were reconstituted in acetonitrile/H_2O (20/80) and analyzed by liquid chromatography (Ultimate 3000 LC system, Dionex, USA)/tandem mass spectrometry (Q Exactive Orbitrap, Thermo Scientific, USA). Samples were analyzed in positive ion mode for morphine, hydromorphone, and morphine-3-glucuronide (M-3-G), the major metabolite of morphine in mice (Kuo et al., 1991). The quantification range for morphine was between 2.0 and 20 000 ng/ml. Hydromorphone was not found in any of the samples.

For ethanol determination, 100 µl plasma samples were mixed through with 500 µl acetonitrile before being dried by speed-vac. Samples were reconstituted in acetonitrile for analysis. Ethanol analysis was then performed using gas chromatography-mass spectrometry (GCMS) and was carried out on a Thermo Finnigan Trace gas chromatograph with a Thermo Finnigan Voyager GCMS.

2.7 Experimental Design & Data Analysis.

Group size was determined by power analysis (G*Power version 3.1.9.2) using previously obtained data (Hill et al., 2016). This indicated a group size of n=7 as being adequate to determine statistical difference between saline and morphine pump treated mice. A single group of mice (control diet fed, saline treated mice) are presented as n = 6 due to a single fatality shortly after the experiment commenced. Also, blood/brain sampling difficulties sometimes provided inadequate sampling for one or both analyzes of morphine and ethanol levels, resulting in only n=6 for these assays. However post-hoc power analyzes confirmed that this remained sufficiently powered.

Area under the curve (AUC) was determined using a 100% baseline as described previously (Hill et al., 2016). Overall changes from a single factor were analyzed using a One-way ANOVA with Bonferroni's post-test. Interaction between prolonged drug treatment and challenge drug was analyzed using a Two-way ANOVA in a two-by-two factorial. Changes in groups over time with repeat measurements were analyzed using a Two-way repeated measures ANOVA with Bonferroni's post-test to analyze drug effect over time. *P* values <0.05 were considered statistically significant. Student's T-tests were used to compare single factor changes. Paired T-tests were used to compare within group changes of a single factor. All T-tests were two-tailed. GraphPad Prism 7 was used for all statistical analyzes. All data are displayed as mean ± standard error of the mean.

2.8 Drugs.

Morphine hydrochloride (Macfarlane Smith) was dissolved in sterile saline and administered i.p. for acute injections or dissolved in distilled water for prolonged

administration using implanted osmotic mini-pumps. Distilled water was used in the mini-pumps due to the lower solubility of morphine in saline. Heparin (Sigma Aldrich UK) was diluted in distilled water to 200 units/ml.

3. RESULTS

3.1. Prolonged ethanol intake and prolonged morphine treatment did not affect diet consumption or body weight

Over the first 8 days of the experiment both control and ethanol fed mice consumed equivalent amounts of the liquid diets (Fig. 1A). There was also no difference in diet consumption in either the control or ethanol fed mice over days 9 – 16 when the mice were treated with saline or morphine (Fig. 1B). However, comparison of morphine treated mice on control diet with those on ethanol diet revealed that the consumption of diet was higher in control than in ethanol diet fed mice (Fig. 1B). Only mice fed on the control diet and treated with saline gained body weight over the 16 days of the experiment; in all other groups/treatments there was no significant change in body weight (Fig. 1C).

3.2. Lack of morphine tolerance in mice fed on ethanol liquid diet.

Following 8 days of control or ethanol diet consumption, respiration was monitored on the morning of day 9 just prior to initiating prolonged morphine treatment (Fig. 2A). There was no difference in minute volume between groups fed on control diet and those fed on ethanol diet. On day 9, mice had their respiration measured prior to and following administration of the first priming dose of morphine (100 mg/kg). Significant respiratory depression was seen in control diet (-47.2% ± 2.8) and ethanol diet (-50.6% ± 3.4) fed mice compared to saline-injected mice where there was no respiratory depression (Fig. 2B & C). This degree of respiratory depression induced by morphine (100 mg/kg) was consistent with that we have previously observed in naïve mice (-52.1% ± 6.3 n=6). Mice continued to be fed on their respective diets and treated for a further 8 days with either saline or morphine. Respiration was measured on days 11 to 16 (i.e. following osmotic mini-pump implantation). Mice fed on control diet that then received prolonged morphine treatment did not show respiratory depression compared to

mice also fed on control diet but treated with saline (Fig. 2D). This indicates that the 3 initial doses of morphine (100 mg/kg i.p.) administered on days 9 and 10 had induced tolerance to morphine depression of respiration such that no respiratory depression in response to morphine released from the osmotic mini-pumps was observed on days 11 to 16. In contrast, in mice that had been fed the ethanol diet, respiration was depressed on days 11 to 16 in the prolonged morphine treated mice but not in the saline treated mice (Fig. 2E). This suggests that prior ethanol exposure either prevents the development of tolerance to morphine or in some other way enhances the effect of morphine thus revealing the respiratory depressant effect of morphine released from the osmotic mini-pumps.

On day 16, all groups of mice received an acute challenge dose of morphine (10 mg/kg i.p.) to determine their sensitivity to morphine depression of respiration. In control and ethanol diet fed mice treated with saline, respiration was depressed by the morphine challenge (Fig. 3A, B and F). The time course and extent of the respiratory depression by morphine were similar for both treatments (40 – 50% depression after 15 – 20 minutes following drug injection). Thus, the consumption of the ethanol diet for 16 days did not enhance the respiratory depressant effects of the acute dose of morphine.

In control diet fed mice that received prolonged morphine treatment the challenge dose of morphine on day 16 did not depress respiration (Fig. 3C, D and F). This demonstrates that the prolonged morphine treated mice were tolerant to the respiratory depressant effects of morphine. In mice that had been fed on the ethanol liquid diet and received prolonged morphine treatment the baseline respiration levels before the morphine challenge were lower than in mice fed the control diet (Fig. 3C and E). When these mice received the challenge dose of morphine on day 16 respiration was further depressed. These data indicate that ethanol diet fed mice did

not develop tolerance during the prolonged morphine treatment and remained fully sensitive to the respiratory depressant effect of the acute morphine challenge.

3.3. Ethanol diet did not alter brain or plasma levels of morphine.

At the end of the morphine challenge experiments (see Fig. 3) mice were sacrificed and blood samples and brains taken for analysis of morphine and ethanol levels. The plasma ethanol levels achieved in ethanol diet fed animals of 208 ± 24 mg/dl (n = 6; Fig 4C) were similar to that reported by Bertola et al. (2013) using the same protocol. Following prolonged morphine treatment the plasma concentration of morphine was 100 ± 12 ng/ml (n = 6; Fig 4A) which is approximately 300 nM. There was no significant difference in either the brain or plasma levels of morphine in mice fed control or ethanol diet followed by prolonged morphine treatment (Fig 4A & B) indicating that ethanol intake had no effect on the distribution and metabolism of morphine. The plasma ethanol concentrations were the same in saline treated and prolonged morphine treated mice (Fig 4C). Hence, the enhanced response to the acute morphine challenge observed in prolonged morphine treated, ethanol fed mice (Fig 3) did not result from higher morphine or ethanol blood/brain levels.

4 DISCUSSION

In the present investigation we observed that prolonged ethanol treatment did not alter the acute respiratory depressant effect of morphine in mice. Morphine depresses respiration by activating of μ opioid receptors as it does not occur in μ receptor knockout mice (Matthes et al., 1996; Romberg et al., 2003). There have been reports that prolonged ethanol treatment can uncouple the μ-opioid receptor from G protein activation (Chen and Lawrence, 2000; Sim-Selley et al., 2002; Saland et al., 2004). However, uncoupling was seen in only some brain regions and not in others and therefore does not seem to be a ubiquitous, consistent response to ethanol treatment across the brain. He and Whistler (He and Whistler, 2011) reported that prolonged administration of high, binge doses of ethanol to rats reduced the antinociceptive response to acute morphine administration but with the ethanol treatment we used we did not observe any reduction in the respiratory depressant effect of morphine.

Importantly, we observed that prolonged ethanol treatment did not alter the respiratory depressant effect of an acute dose of morphine. This would indicate that the ethanol treatment had not altered the metabolism or distribution of morphine. However, prolonged ethanol treatment did prevent the development of tolerance to morphine-induced respiratory depression. This inhibition of tolerance development extends our previous work in which we demonstrated that acute administration of a relatively low dose of ethanol reversed tolerance to morphine-induced respiratory depression (Hill et al., 2016) and antinociception (Hull et al., 2013). Furthermore, we have previously reported that a mildly intoxicating concentration of ethanol in humans (20 mM) reversed morphine-induced cellular tolerance in brain neurons (Llorente et al., 2013). If a similar phenomenon is observed in humans then ethanol reversal of tolerance would have two important consequences. First, consumption of alcohol by heroin

users would result in a lower level of opioid tolerance, increasing the respiratory depressant response to heroin injection – heroin is rapidly converted to 6-monoacetylmorphine (6MAM) and then to morphine – and so increasing the likelihood of overdose and death. Second, between heroin injections users begin to experience symptoms of withdrawal increasing the drive to retake the drug. A reduced level of tolerance induced by alcohol consumption would enable low, residual levels of morphine in the body to activate the μ-opioid receptor which might help to stave off the experience of withdrawal.

With prolonged activation the μ-opioid receptor desensitizes, and this desensitization contributes to the level of tolerance (Williams et al., 2013). The mechanisms of μ-opioid receptor desensitization have been shown to be agonist-specific (Kelly et al., 2008), with good evidence to suggest that protein kinase C (PKC) mediates μ receptor desensitization and tolerance induced by morphine, a relatively low efficacy agonist at the μ-opioid receptor whereas G protein-coupled receptor kinases (GRKs) and arrestin binding mediates desensitization and tolerance induced by high efficacy μ receptor agonists (Williams et al., 2013). In our studies on tolerance to opioid-induced respiratory depression PKC inhibition reversed tolerance induced by prolonged morphine and oxycodone treatments but not tolerance induced by the high efficacy agonist methadone (Withey et al., 2017). One possibility is that ethanol may in some way block the PKC mechanism known to underlie tolerance to morphine and other low efficacy μ-opioid agonists. Direct inhibition of conventional PKC isoforms by ethanol has been difficult to demonstrate, with only modest inhibition observed at high ethanol concentrations (see Llorente et al., 2013 for discussion). It is also possible that ethanol does not directly inhibit PKC activity but prevents PKC translocation to the receptors on the plasma membrane or that acetaldehyde rather than ethanol inhibits PKC (Domenicotti et al., 1998).

At post mortem (autopsy) the presence of alcohol is more frequently detected in heroin-related deaths than in those involving methadone (Bryant et al., 2004; Shah et al., 2005). This might relate to the ability of ethanol to reverse morphine but not methadone tolerance. In those deaths in which ethanol and methadone were found to be present at post mortem additivity of effect to depress respiration at high drug levels, rather than reversal of tolerance, is more likely to be the cause of death.

We have previously reported that in mice, tolerance to respiratory depression induced by prolonged methadone treatment is not reversed by ethanol or PKC inhibitors (Hill et al., 2016; Withey et al., 2017). Methadone has higher agonist efficacy than morphine at the μ-opioid receptor (McPherson et al., 2010). If ethanol only reverses PKC-dependent tolerance, then consumption of alcohol would not reduce tolerance to the respiratory depressant effects of methadone.

4.1 CONCLUSION

Our study highlights the danger of poly drug abuse by heroin users and expands upon our previous work on ethanol reversal of tolerance to morphine respiratory depression. Here we clearly demonstrate that prior, prolonged exposure to ethanol prevents the development of tolerance to morphine respiratory depression. Thus in humans alcohol consumption will increase the likelihood of fatal overdose occurring with doses of heroin or morphine that might not otherwise have been expected to be fatal.

Keywords:

Overdose

Ethanol

Morphine

Tolerance

Respiratory depression

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FIGURE LEGENDS

Fig. 1. Consumption of liquid diets and mouse body weight.

(A) Consumption of control or ethanol liquid diet per day (Days 1-8) by mice prior to treatment with saline or morphine. No significant difference in diet consumption was found between groups. **(B)** Consumption of liquid diet per day (Days 9-16) by mice during prolonged treatment with saline or morphine. Morphine treated, ethanol diet fed mice did not consume less ethanol diet than saline treated, ethanol diet fed mice, but did consume less than morphine treated, control diet fed mice. Data in **A** & **B** are calculated by group consumption and so cannot be presented as individual data points. **(C)** Weight of mice on day 0 and day 16 on liquid diets. Only mice on the control liquid diet that received saline treatment for the last 8 days gained weight significantly. No other group of mice significantly lost or gained weight over the experimental period. In (**A)** [$F = 0.657$ ($dfn = 3$, $dfd = 23$)] and **(B)** [$F = 2.92$ ($dfn = 3$, $dfd = 23$)] mice were compared by One-way ANOVA with Bonferroni's comparison. In **C** beginning and end weights were compared by a two-tailed paired Student's T-test [$t = 5.162$ ($dfd = 6$)]. * indicates $p<0.05$; N=7 for all groups except for control diet fed saline treatment mice where n=6.

Fig. 2. Effect of ethanol liquid diet on the development of tolerance to morphine depression of respiration.

(A) Mice from all 4 groups did not have significantly different baseline respiration, measured as minute volume prior to saline or morphine treatment [$F = 1.26$ ($dfn = 3$, $dfd = 18$)]. The same data are reproduced as 'Day 9' data points in **(D)** and **(E).** (**B & C**) Acute administration of the first priming dose of morphine (100 mg/kg) to control [$F = 27.85$ ($dfn = 10$, $dfd = 132$)] or ethanol [$F = 31.80$ ($dfn = 10$, $dfd = 132$)] diet fed mice induced significant respiratory

depression compared to saline administered controls. (**D**) Prolonged morphine treatment beginning on day 9 did not depress respiration in mice fed control liquid diet. (**E**) Prolonged morphine treatment beginning on day 9 did result in depression of respiration in mice fed the ethanol liquid diet [$F = 6.68$ (dfn = 6, dfd = 84)]. All mice were on their respective diets for 8 days prior to the saline and morphine treatments. Respiration was measured as minute volume. * indicates $p<0.05$. Groups compared by Two-way ANOVA with Bonferroni's comparison. N=7 for all groups except for control diet fed saline treatment mice where n=6.

Fig. 3. Effect of ethanol liquid diet on the expression of morphine tolerance.

In **(A)** and **(C)**, data are presented as minute volume (MV) whereas in **(B)** and **(D)**, the data have been transformed such that the level of respiratory depression seen following morphine challenge is expressed as a percentage change in the pre-challenge MV baseline, calculated for each mouse individually before mean data were plotted. In **(F)** the data in **(B)** and **(D)** have been recalculated and plotted as the area under the curve (AUC). The AUC was calculated for each individual mouse (data points shown) before the mean AUC has been calculated (open histograms). In **(A)** and **(B)** acute morphine challenge (10 mg/kg i.p.) depressed respiration in mice fed the ethanol or control liquid diet and treated with saline for the last 8 days. In **(C)** and **(D)** morphine challenge did not cause a decrease in MV in mice fed control liquid diet and pre-treated with morphine for the last 8 days, but did cause a decrease in MV in mice morphine pre-treated mice fed ethanol liquid diet. In **(E)** baseline respiration prior to acute morphine treatment is shown (calculated from time 10-20 min in **(A)** and **(C)**). Only ethanol diet fed and morphine treated mice had significantly reduced baseline respiration. In **(E)** [$F = 16.89$ (dfn = 3, dfd = 15)] and **(F)** [$F = 11.24$ (dfn = 3, dfd = 15)] groups were compared by Two-way ANOVA with Bonferroni's comparison. In **C** * indicates comparison to control diet fed

mice prior to morphine challenge [F = 11.58 (dfn = 10, dfd = 132)]. * Indicates $p<0.05$; N=7 for all groups except for control diet fed, saline treatment mice, where n=6.

Fig. 4. Ethanol and morphine levels following prolonged ethanol liquid diet.

Plasma **(A)** [t = 0.35 (dfn = 10)] and brain **(B)** [t = 0.25 (dfn = 10)] levels of morphine in mice treated with morphine for the last 8 days. There was no significant difference in plasma or brain levels of morphine in mice fed control or ethanol liquid diet. **(C)** [t = 0.06 (dfn = 10)] Plasma ethanol levels in mice fed ethanol liquid diet for 16 days. There was no significant difference in plasma ethanol levels between ethanol liquid diet fed mice treated with saline or morphine for the last 8 days. Groups compared by an unpaired two-tailed Student's t-test. N=6 for all groups.

Figure 1

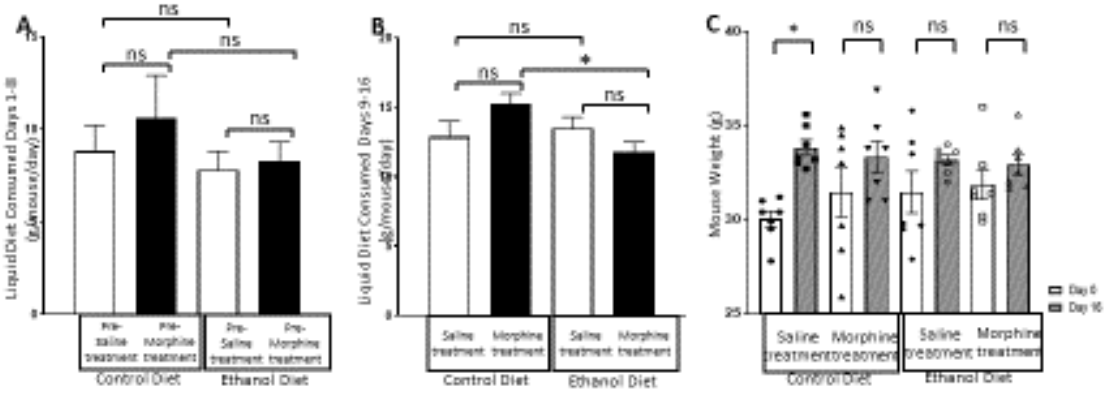
A
B
C
ns
*
Pre-saline treatment
Pre-morphine treatment
Pre-Saline treatment
Pre-Morphine treatment
Saline treatment
Morphine treatment
Control Diet
Ethanol Diet
Mouse Weight (g)
Day 6
Day 16

Figure 2

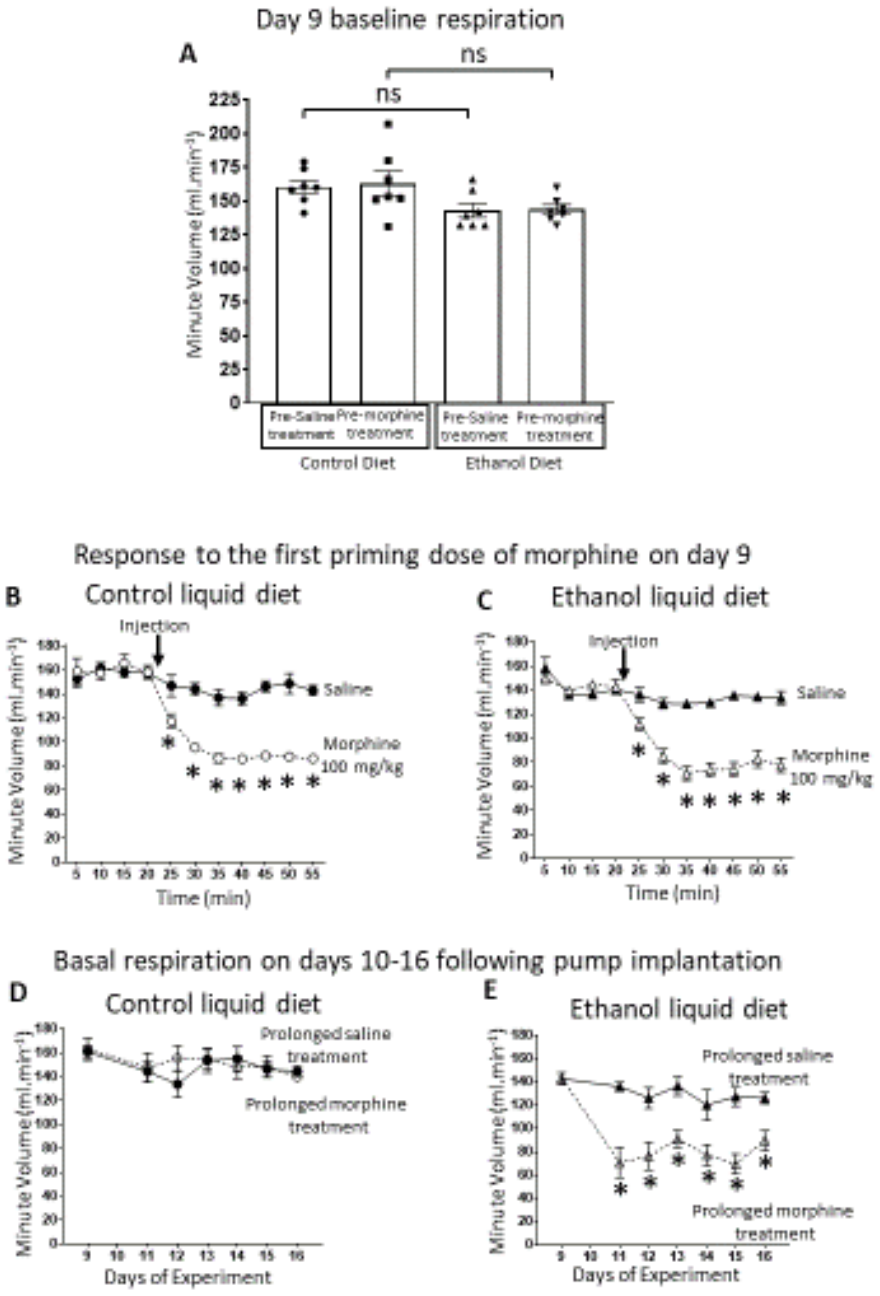
Day 9 baseline respiration
A
ns
ns
Minute Volume (ml.min-1)
Pre-Saline treatment
Pre-morphine treatment
Pre-Saline treatment
Pre-morphine treatment
Control Diet
Ethanol Diet
Response to the first priming dose of morphine on day 9
B
Control liquid diet
Injection
Saline
Morphine 100 mg/kg
Time (min)
C
Ethanol liquid diet
Injection
Saline
Morphine 100 mg/kg
Time (min)
Basal respiration on days 10-16 following pump implantation
D
Control liquid diet
Prolonged saline treatment
Prolonged morphine treatment
Days of Experiment
E
Ethanol liquid diet
Prolonged saline treatment
Prolonged morphine treatment
Days of Experiment

Figure 3

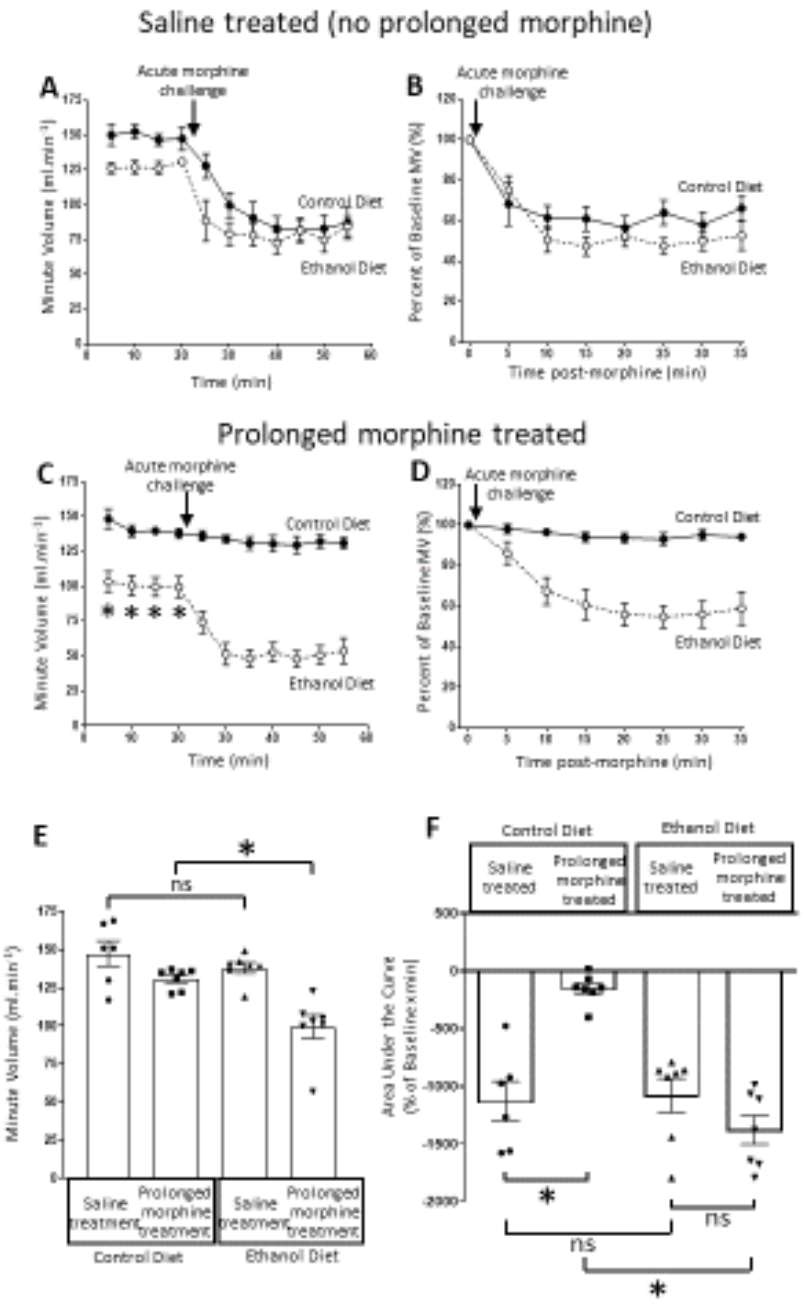
Saline treated (no prolonged morphine)
A
Acute morphine challenge
Minute Volume [ml.min⁻¹]
Control Diet
Ethanol Diet
Time (min)
B
Acute morphine challenge
Percent of Baseline MV (%)
Control Diet
Ethanol Diet
Time post-morphine (min)
Prolonged morphine treated
C
Acute morphine challenge
Minute Volume [ml.min⁻¹]
Control Diet
Ethanol Diet
Time (min)
D
Acute morphine challenge
Percent of Baseline MV (%)
Control Diet
Ethanol Diet
Time post-morphine (min)
E
*
ns
Minute Volume (ml.min⁻¹)
Saline treatment
Prolonged morphine treatment
Saline treatment
Prolonged morphine treatment
Control Diet
Ethanol Diet
F
Control Diet
Ethanol Diet
Saline treated
Prolonged morphine treated
Saline treated
Prolonged morphine treated
Area Under the Curve (% of Baseline x min)
*
ns
ns
*

Figure 4


Plasma Morphine
A
ns
Plasma Morphine (ng/ml)
175
150
125
100
75
50
25
0
Control Diet
Ethanol Diet
Brain Morphine
B
ns
Brain Morphine (ng/g)
25
20
15
10
5
0
Control Diet
Ethanol Diet
Plasma Ethanol
C
ns
Plasma Ethanol (mg/dL)
400
300
200
100
0
Saline treated
Prolonged morphine treated